MASS SPECTROMETRIC DETECTION OF SULFUR MUSTARD ADDUCTS TO PROTEINS AND DNA: DOSIMETRY OF EXPOSURE TO SULFUR MUSTARD

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ABSTRACT

Immunochemical detection methods have been developed for analysis of protein and DNA adducts of sulfur mustard within our studies performed in collaboration with TNO-Medical Biological Laboratory. In order to validate immunochemical determinations, mass spectrometric detection methods are being developed as an independent, highly sensitive technique having an almost absolute specificity. A method has been developed for specific cleavage of the alkylated N-terminal valine in the α-chain of human hemoglobin exposed to sulfur mustard, by using the modified Edman reagent pentafluorophenyl iso-thiocyanate. After subsequent reaction with heptafluorobutyric anhydride, the derivatized alkylated valine is determined by gas chromatography/negative chemical ionization-mass spectrometry (GC/NCI-MS). In a second approach, a procedure for analysis of the major amino acid adduct formed upon alkylation of hemoglobin with sulfur mustard will be developed. To this end, the protein is alkylated with ^{35}S-sulfur mustard and is subsequently digested by a proteolytic enzyme. After appropriate derivatization of the (alkylated) amino acids in the digest, HPLC analyses with radiometric detection and spiking with synthesized reference adducts and GC/MS analyses are performed in order to show which alkylated amino acids have been formed, and survive digestion. Furthermore, adducted peptides were identified by LC-MS analysis in a trypsin digest of the alkylated globin.

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Introduction

The confirmed use of mustard gas, sarin and tabun in the Iran-Iraq conflict and the threat of chemical warfare in the recent Gulf War have stressed the need of reliable methods to detect the nature and extent of poisoning with chemical warfare agents.

In this context, we developed immunochemical analysis procedures for sulfur mustard adducts to DNA which allow to detect exposure of human blood to 2 μM sulfur mustard and exposure of human skin to sulfur mustard vapor at Ct-values that do not cause blister formation (1). Upon exposure of human blood to sulfur mustard, we found much more agent bound to hemoglobin and other proteins than to the much less abundant DNA of the white cells. We did not succeed so far in developing a suitable immunochemical method for detection of sulfur mustard-protein adducts.

Presently, we are extending these investigations by improving immunochemical detection methods, which will be reported separately (2), and by developing independent analysis methods. Independent procedures that are absolutely specific are of the utmost importance to validate the immunochemical methods. Moreover, instrumental analysis may provide lower detection limits than immunochemical methods, yielding unequivocal results in cases where the immunochemical results are indecisive. In this approach, adducts are derivatized with electrophore-labels which renders the derivatives sufficiently volatile for GC/MS and provides moieties which capture low energy electrons thereby allowing highly sensitive detection in negative chemical ionization mass spectrometry (NCI-MS). In this communication we present qualitative analysis results obtained with methods that have been developed so far for determination of sulfur mustard adducts with hemoglobin.

Experimental part

[^{35}S]sulfur mustard was prepared from ethylene oxide and hydrogen [^{35}S]sulfide obtained by reduction of [^{35}]sulfate with hydrogen iodide, formic acid and sodium phosphite, and by subsequent chlorination of the formed [^{35}]thiodiglycol with thionylchloride. Analogously, [U-^{2}H]sulfur mustard was obtained starting from [U-^{2}H]ethylene oxide and hydrogen sulfide. The amino acid adducts were prepared by alkylation of cysteine with 2-hydroxyethyl-2'chloroethyl sulfide and of valine and N_{α}-Boc-histidine with 2-acetoxyethyl-2'chloroethyl sulfide, by esterification of the N-Boc-glutamic acid and N-Boc-aspartic acid 1-t-butyl esters with thiodiglycol using dicyclohexylcarbodiimide as a coupling reagents. N^{im}-(2'-hydroxyethylthio-ethyl)-his (N^{im}-HETE-his), 5-HETE-glu and 4-HETE-asp were obtained after subsequent removal of the protecting groups with trifluoroacetic acid. Pronase E was obtained from Sigma Chemical Comp. (St. Louis, MO).

GC/MS spectra were recorded on a VG70-250s mass spectrometer coupled to a HP 5890A gas chromatograph and a Unicam Automass 150 coupled to a Carlo Erba Mega 5300 gas chromatograph. LC/thermospray-MS spectra were recorded on a Nermag R10-10C quadrupole instrument, equipped with a TSP ion source, which was coupled with the LC system via a Vestec TSP interface. Fast atom bombardment (FAB)-MS/MS was performed on a VG Autospec T tandem sector (EBE-BE) instrument with a focal plane detector. The samples determined in the

latter instrument were solutions in thioglycerol or in glycerol containing 50 % m-nitrobenzyl alcohol and 1 % trifluoroacetic acid.

Results and Discussion

Feasibility of modified Edman degradation for analysis of N-terminal valine in the Á-chain of hemoglobin alkylated by sulfur mustard

In our previous studies (1) we established that the N-terminal valine of the α-chain in human hemoglobin is alkylated upon exposure of the protein to sulfur mustard. Tornqvist et al. (3) have developed a specific method for analysis of an alkylated N-terminal valine in hemoglobin, which is cleaved off by the modified Edman sequencing reagent pentafluorophenyl isothiocyanate in the neutral coupling medium of the reaction. In this way the tedious complete hydrolysis of the alkylated hemoglobin can be avoided.

We tested the feasibility of this method for analysis of the alkylated N-terminal valine in hemoglobin upon exposure of human blood to sulfur mustard. After isolation of alkylated globin and treatment with the modified Edman reagent, the isolated analyte was derivatized with heptafluorobutyric anhydride (HFBA). GC/NCI-MS analysis showed the spectrum of the expected product (Figure 1) as confirmed by analysis of N-HETE-val derivatized in the same manner.

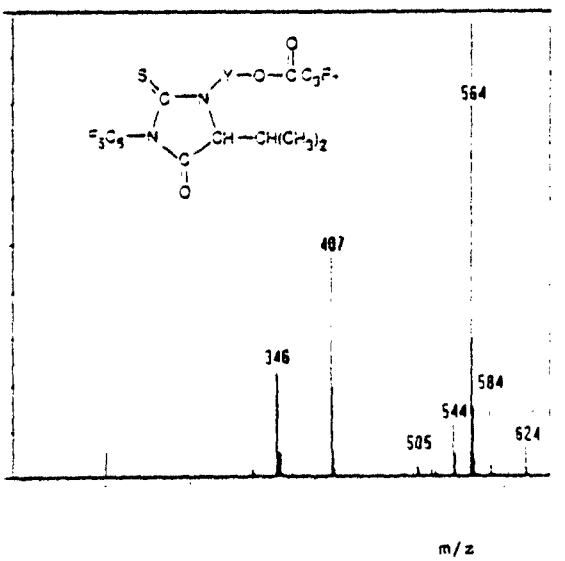

Figure 1. NCI-MS spectrum of N-pentafluorophenylthiohydantoin-N-(2'-heptafluorobutyroxyethylthioethyl)-valine; Y = $CH_2CH_2SCH_2CH_2$

Similar results were obtained for alkylated globin obtained from human blood exposed to [U-^{2}H]sulfur mustard. Globin alkylated with [U-^{2}H]sulfur mustard may serve as an internal standard in a quantitative analysis which will be developed according to this procedure.

Identification of amino acid adducts formed in globin upon exposure to sulfur mustard

In the last decade, several groups of investigators have developed methods for GC/NCI-MS analysis of alkylated amino acids in proteins, as an internal dosimeter for exposure to alkylating agents (4). Usually, hemoglobin is selected as the protein for analysis, since it is easily available and has a half-life of ca. 120 day in humans, which allows to detect an integrated dose over a prolonged period of time. Briefly, protein adducts are determined by acidic hydrolysis (6 M HCl) of the protein, derivatization of the alkylated amino acids and GC/MS analysis.

It is anticipated that sulfur mustard-amino acid adducts may be acid-labile, particularly the adducts formed with glutamic and aspartic acid. Therefore, enzymatic hydrolysis on sulfur mustard adducts of globin by pronase E was applied, in addition to acidic hydrolysis.

Two approaches were followed to identify sulfur mustard adducts formed in human globin: HPLC analysis of [^{35}]sulfur mustard adducts and GC/MS analysis.

Derivatization of (alkylated) amino acids in the hydrolysates is necessary for a proper analysis by both techniques. Reaction of 9-fluorenylmethyl chloroformate (FMOC-Cl) at N_δ of at the (alkylated) amino acid yielded derivatives suitable for analysis by HPLC. For GC/MS analysis, (alkylated) amino acid were volatilized by

(i) acylation with HFBA at N_α and the hydroxyl group of the adduct moiety, followed by esterification of the carboxyl moiety with methanol/HCl, or by

(ii) cyclization of the α-amino and the carboxyl moieties with dichlorotetrafluoroacetone (DCTFA, ref. 5) followed by acylation of the adduct hydroxyl group with HFBA (see Figure 2 for an example).

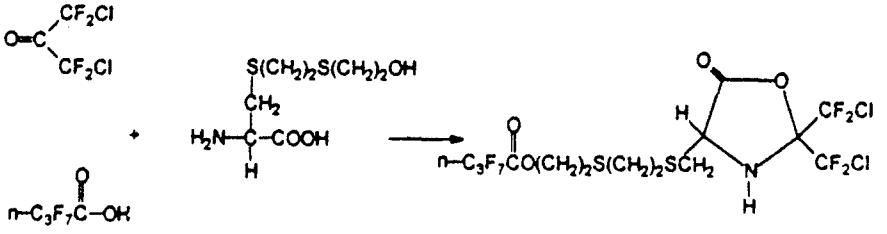

Figure 2. Reaction scheme for derivatization of S-HETE-cys with dichlorotetrafluoroacetone (DCTFA) and heptafluorobutyric anhydride (HFBA)

These derivatization procedures introduce electrophore-labels which allow highly sensitive detection by NCI-MS.

The pronase E digest of human globin treated with [^{35}S]sulfur mustard (1 mM) was analyzed with HPLC after derivatization with FMOC-chloride. Radiometric detection revealed three radioactive peaks (Figure 3). Based on coelution with the synthesized reference compounds, the peaks were tentatively assigned to the FMOC derivatives of N^{im}-HETE-his (peak 1), of 5-HETE-glu and/or 4-HETE-asp, which have a similar elution profile (peak 2), and of S-HETE-cys (peak 3). It was calculated that the radioactive peaks found for N^{im}-HETE-his, 5-HETE-glu and/or 4-HETE-asp and S-HETE-cys correspond with 26, 60 and 40 nmol adduct/g globin. N-HETE-val was not detected which might be due to the sluggish reaction of the secondary nitrogen of N-HETE-val with FMOC-chloride.

Upon acidic hydrolysis of globin treated with [^{35}S]sulfur mustard (75 mM), a radioactive peak corresponding with 9.6 µmol N^{im}-HETE-his/g globin was detected in the hydrolysate, after FMOC-chloride derivatization. As expected, the esterified products 5-HETE-glu and/or 4-HETE-asp were not detected in an acidic hydrolysate of human globin treated with [^{35}S]sulfur mustard (75 mM). Moreover, S-HETE-cys was not detected in this hydrolysate.

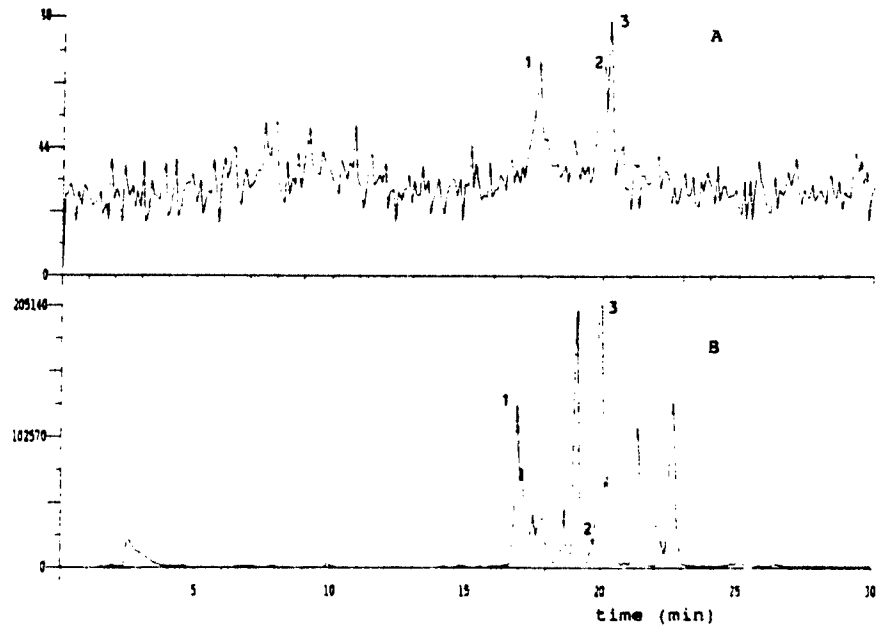

Figure 3. HPLC chromatogram (PEP-RPC 5/5 column) of pronase E digest of human globin treated with [^{35}S]sulfur mustard (1 mM), after derivatization with FMOC-chloride; A, detection of radioactivity; B, UV detection. FMOC derivatized S-HETE-cys was coeluted. An acetonitrile gradient (0 - 70 %) in water containing 0.1 % trifluoroacetic acid was used as an eluent. Peak 1, N^{im}-HETE-his; peak 2, 5-HETE-glu and/or 4-HETE-asp; peak 3, S-HETE-cys.

Final identification of the adducts was achieved by GC/MS analysis of the enzymatic and acidic hydrolysates by monitoring for a distinctive mass fragment of the adduct. The analyte was further identified by comparison of the retention time at which the fragment was detected and of the mass spectrum scanned at that particular retention time with those of the synthesized reference compounds. 5-HETE-glu, 4-HETE-asp and S-HETE-cys were identified with GC/NCI-MS analysis in the pronase digest after derivatization with DCTFA-HFBA, whereas N^{im}-HETE-his was identified with GC/EI-MS after derivatization of the digest with HFBA-methanol/HCl. N-HETE-val was identified in the acidic hydrolysate with GC/EI-MS after derivatization with DCTFA-HFBA.

Identification of alkylation sites in hemoglobin

The development of "soft" ionization techniques such as fast atom bombardment (FAB) has made it possible to determine with high accuracy and high sensitivity the molecular weight of peptides up to 2000. Hybridization of various analytical techniques, *e.g.*, of FAB-MS with HPLC or capillary electrophoresis allows the on-line determination of the molecular weights of, *e.g.*, mixtures of peptides resulting from tryptic digestion of proteins. Advances in tandem mass spectrometry allow the sequencing of such peptides containing up to 25-30 amino acids.

In the present work we are investigating the feasibility of these techniques for analysis of hemoglobin alkylated by sulfur mustard. A scientific bonus of the mass spectrometric analysis of alkylated peptides is the identification of the site of alkylation by sulfur mustard within the tertiary structure of hemoglobin. So far, we performed (i) preliminary LC/thermospray-MS analyses in a tryptic digest of globin treated with sulfur mustard (100 mM) and (ii) a determination of the amino acid sequence in the synthesized N-terminal neptapeptide of the α-chain in hemoglobin alkylated with sulfur mustard, from FAB-MS/MS analysis.

Since the structures of the peptides formed from hemoglobin upon tryptic digestion are known, ion chromatograms of the possible protonated or doubly protonated peptides formed and modified with a HETE moiety were constructed after LC/thermospray-MS analysis of a tryptic digest from hemoglobin treated with sulfur mustard. Three peptide adducts were identified so far, all from the α-chain of hemoglobin:

HETE-val-leu-ser-pro-ala-asp-lys [HETE-T1, m/z 833 (MH^+)],
HETE-(val-gly-ala-his-ala-gly-glu-tyr-gly-ala-glu-ala-leu-glu-arg) [HETE-T4, m/z 817 (MH_2^{++})], and
HETE-(val-asp-pro-val-asn-phe-lys) [HETE-T11, m/z 922 (MH^+)].

The structure of HETE-T1, *i.e.*, the N-terminal heptapeptide of the α-chain alkylated at N-terminal val, was confirmed by comparison of its retention time and mass spectrum with those of the synthetic product.

The amino acid sequences of synthesized HETE-T1 and of the parent heptapeptide were determined with FAB-MS/MS analysis. The mass spectra obtained (Figure 4) confirm the structures of the peptides and N-terminal valine as the site of alkylation by sulfur mustard. In accordance with alkylation at the N-terminal amino acid, the C-terminal fragments Y1 to Y6 are identical for the peptides, whereas the m/z values for all N-terminal B fragments differ 104, *i.e.*, the molecular mass of the HETE moiety. Clear

evidence for the site of alkylation is also obtained from the m/z value for the A1 fragment, $[HETE\text{-}NHCH(C_3H_7)]^+$, in the spectrum of the alkylated heptapeptide.

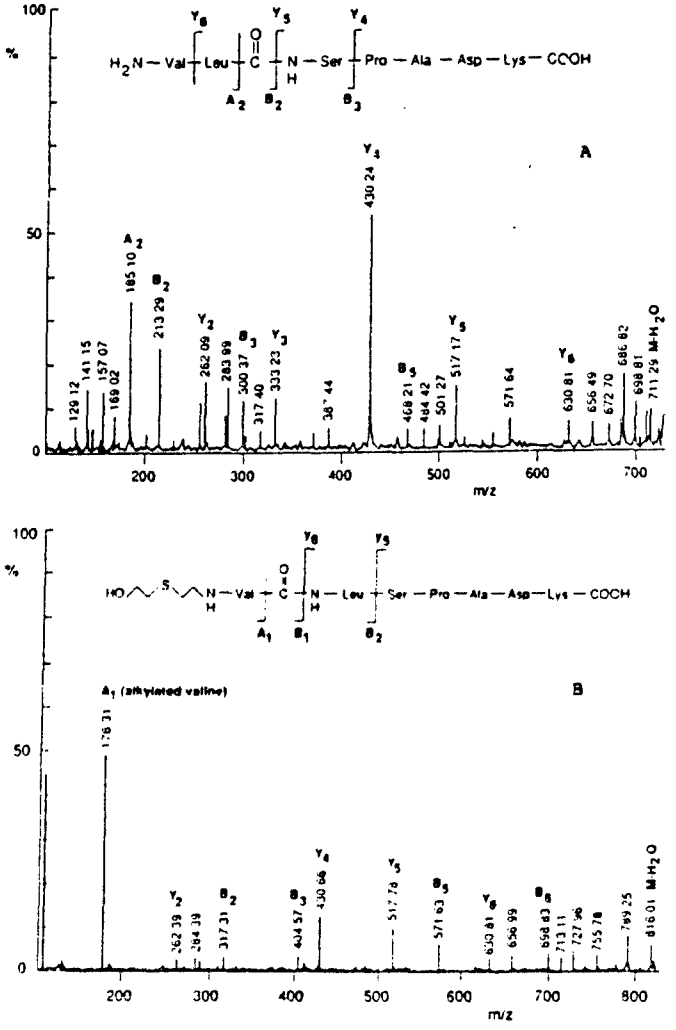

Figure 4. FAB-MS/MS of the synthesized N-terminal heptapeptide of the α-chain in hemoglobin (A) and of this peptide treated with sulfur mustard (B)

Conclusions

Derivatization methods were developed for analysis of sulfur mustard-amino acid adducts with HPLC, GC and GC/(NCI-)MS.

N-HETE-val, N^{im}-HETE-his, S-HETE-cys, 5-HETE-glu and 4-HETE-asp were identified in enzymatic and/or acidic hydrolysates of globin exposed to sulfur mustard.

The adducts of val and his are reasonably stable to acidic hydrolysis.

The modified Edman degradation can be used for determination of the sulfur mustard adduct formed with N-terminal val in the α-chain of hemoglobin.

Three peptide adducts could be detected so far by LC/thermospray-MS in tryptic digests of globin exposed to sulfur mustard; one of these peptide adducts was identified as the N-terminal heptapeptide from the α-chain alkylated at the N-terminal val.

The site of alkylated by sulfur mustard of the synthesized N-terminal heptapeptide from the α-chain of hemoglobin could be established by FAB-MS/MS.

Results obtained allow the development of procedures for sensitive determination of sulfur mustard adducts to hemoglobin by using GC/NCI-MS and for identification of sites of alkylation in hemoglobin by using FAB-MS/MS.

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